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Assay of Chicken Brain Neurotoxic Esterase Activity Using Leptophosoxon as the Selective Neurotoxic Inhibitor

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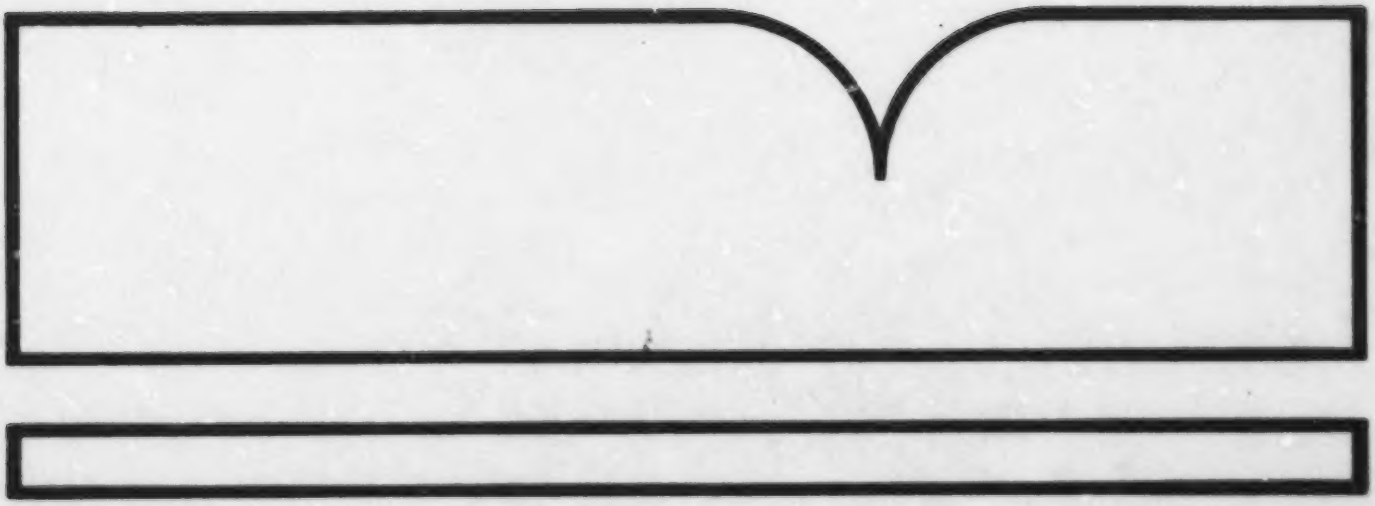

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16. ABSTRACT

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Journal Article

Assay of Chicken Brain Neurotoxic Esterase Activity Using Leptophosoxon as the Selective Neurotoxic Inhibitor

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Abstract

Hen brain microsomal preparation has phenyl valerate-hydrolyzing activity associated with neurotoxic esterase activity. Part of that activity is due to paraoxon-insensitive esterases and a sub-part of this is sensitive to neurotoxic organophosphates, *i.e.*, mipafox and leptophosoxon. This neurotoxic agent sensitive esterase activity is referred to as neurotoxic esterase (NTE). Because of the commercial unavailability and high toxicity of mipafox, which is usually used as the selective inhibitor for assaying NTE, leptophosoxon was used as an alternative to mipafox. Results indicated that the NTE fraction of hen brain microsomal PV-hydrolyzing activity is the same target for either mipafox or leptophosoxon. The inhibitory effect of leptophosoxon against that fraction was much higher than that of mipafox. The availability of leptophos/leptophosoxon makes this assay very useful for screening organophosphorus esters for neurotoxic effects.

Introduction

Delayed neuropathy, or "toxic distal axonopathy" (1) is one of the most serious effects of some organophosshorus (OP) compounds. This special toxic neuropathy is accompanied by axonal degeneration and demyelination of both the central and peripheral nerve axons of certain species of vertebrates (2). Briefly, in man, chicken, and cat, the most sensitive species, there is a delay period of 8-12 days after exposure, before structural changes occur in the nervous system (3-6).

Because of the phosphorylating property of the OP compounds, it is believed that these compounds produce their biological effects through a mechanism in which phosphorylation of subcellular component(s) occurs. Proteins, and particularly esterases, bind very avidly with OP esters (7). Thus, it is natural to suppose that the neurotoxic lesion, that is induced by OP compounds, may arise from the phosphorylation of a protein or the inhibition of an esterase in the nervous system. Actually, many neurotoxic OP esters are specific inhibitors of cholinesterase (ChE) rather than acetylcholinesterase (AChE). However, the reverse is not always the case: The selective inhibitors of the enzyme (ChE) involve some non-neurotoxic OP compounds such as tetraisopropyl pryophosphate or iso-OMPA, N,N'diisopropylphosphorodiamidic anhydride (8). Moreover, there is no apparent relationship between ChE inhibition and neuropathy induced by OP compounds (9). On the other hand, there appears to be a relationship between the neuropathic effect of OP compounds and their reaction with a brain protein in hens (10-14). This reaction was reported as the phosphorylation of the active site of an esterase referred to as neurotoxic esterase (NTE) which hydrolyzes phenylphenylacetate (PPA) or phenylvalerate (PV) and can be distinguished analytically from other PPA- or PV-hydrolyzing esterases by its response to inhibitors (15,16). *In vivo* studies with 12 neurotoxic OP esters and 11 non-neurotoxic analogs showed a good correlation between the development of the morphologic lesion and the early disappearance of the brain NTE activity of the treated hens (13). *In vitro* tests with more than 100 organophosphorus compounds showed that the neurotoxic chemical, or at least one of its metabolites, must be a potent NTE inhibitor (17,18).

An improved, indirect, differential assay of NTE for screening OP compounds for neuropathy using PV as a substrate was reported (19). A direct technique to assay NTE activity using 4-nitrophenyl valerate (4-NPV) as substrate was recently developed (20). The cornerstone in these methods for the assay of NTE activity is to distinguish its activity from other PV- or 4-NPV-hydrolyzing esterases by its response to the selective inhibitor, mipafox (N,N'-diisopropylphosphorodiamidic fluoride). Mipafox is no longer commercially

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available, but it can be synthesized with special care (19,21). During the Second International Congress on Toxicology, Brussels, July 6-11, 1980, some investigators criticized the use of the NTE assay for screening OP esters for neuropathy because of the difficulty in obtaining mipafox. The aim of this study is to investigate the possibility of using another neurotoxic OP compound, leptophosoxon [0-methyl-0-(4-bromo-2,5-dichlorophenyl) phenylphosphonate] as the selective inhibitor in order to identify and assay for NTE activity.

Materials and Methods

Paraoxon [0,0-diethyl-0-(4-nitrophenyl) phosphate] was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Mipafox and phenylvalerate (PV) were synthesized as previously reported (21). Leptophosoxon, analytically pure, was obtained from the Quality Assurance Section, Analytical Chemistry Branch, Research Triangle Park, North Carolina. All the other chemicals used were of the highest purity grade available from Sigma Chemical Company, St. Louis, Missouri, and/or Aldrich Chemical Company.

Preparation of the Chicken Brain Microsomal Fraction

Normal, adult, white leghorn hens (1.5-2.0 kg) were obtained from Featherdown Farm, Apex, North Carolina. Hens were sacrificed by decapitation. Brains were quickly excised and homogenized as 1:10 (w/v) with *tris*-HCl buffer (50 m*M* pH 8.0) containing 0.2 m*M* EDTA for 20 seconds using a Polytron homogenizer with the suitable titanium bits. The microsomal fraction was isolated by differential centrifugation using a Beckman preparative ultracentrifuge (L5-50) with the appropriate rotors. The microsomal pellets were resuspended in the appropriate volume of *tris*-buffer in order to obtain a suspension equivalent to 3 g of the original brain tissue in 10 mL. Temperature was kept between 0-4°C during these preparations. The protein content of the resultant microsomal preparation was determined by the method of Lowry (22).

Determination of the Total PV-Hydrolyzing Activity

The total PV-hydrolyzing activity of the hen brain microsomal preparation was determined at 37°C. The incubation mixture contained 100 μL of the microsomal preparation and 20 μL of a dimethylformamidic solution of phenylvalerate (25 mg/mL) in a final volume of 3 mL with *tris*-HCl buffer. The reaction was terminated after 10 minutes by the addition of 2 mL of 1% sodium dodecyl sulfate solution containing 0.025% 4-aminoantipyrine (in the same buffer). The liberated phenol was determined colorimetrically after the addition of 1 mL of 0.4% aqueous solution of potassium ferricyanide by recording the intensity of the red color at 510 nm as a measure of the activity against a blank from which phenylvalerate was omitted.

Assay of NTE Activity

The NTE activity of the chicken brain microsomal fractions and the effect of different concentrations of mipafox and leptophosoxon on that activity was determined using the Johnson differential technique (19) with slight modifications. Paired samples of the microsomal fraction (200 μL) were preincubated in *tris*-HCl buffer for 20 minutes at 37°C with paraoxon (40 μ*M*) plus either (a) buffer or (b) the tested concentration of mipafox or leptophosoxon in a final volume of 3 mL. Paraoxon and mipafox were prepared in *tris*-citrate buffer (50 m*M*, pH 6.0) to obtain solutions from which 200 μL gave the tested concentration when added to the final enzymatic reaction mixture (3.0 mL). An acetone solution of leptophosoxon (11.88 mg/20 mL) was prepared and used as stock solution to prepare a series of concentrations, by diluting with *tris*-citrate buffer, from which 200 μL gave the test concentrations (0.1 to 10 μ*M*) when added to the final enzymatic reaction mixture (3 mL). The maximum residual amount of acetone has no effect on PV-hydrolyzing activity. At the end of the preincubation periods, 20 μL of the dimethylformamidic solution of PV was added and the incubation was continued for 10 minutes. The enzymatic reaction was stopped at the end of the incubation period by adding 2 mL of the sodium dodecyl sulfate solution and the tube contents were mixed immediately. One milliliter of the aqueous potassium ferricyanide solution was then added and after 10 minutes, the resultant stable red color was recorded at 510 nm against corresponding blanks from which PV was omitted. Readings were corrected by subtraction of the non-enzymatic hydrolysis of the PV solution used, which was determined in the same manner in the absence of the microsomal fraction. The corrected readings of tubes "a" reflected the remaining paraoxon-insensitive PV-hydrolyzing activity while those of tubes "b" reflected the remaining paraoxon plus mipafox- or leptophosoxon-insensitive PV-hydrolyzing activity at the tested concentrations.

Results indicated that about 30% of the remaining paraoxon-insensitive PV-hydrolyzing activity was insensitive to mipafox or leptophosoxon, even at relatively high concentrations. Neurotoxic esterase activity was calculated using this differential technique by subtraction of that remaining paraoxon plus mipafox- or leptophosoxon-insensitive PV-hydrolyzing activity from the remaining paraoxon-insensitive activity. The whole experiment was repeated three times, each time using fresh preparations. The acetonic solution of leptophosoxon was found to be stable and lost no activity by storing in the freezer (-10°C) for four weeks.

Results

Total and Paraoxon-Insensitive Hen Brain PV-hydrolyzing Activity

The PV-hydrolyzing total specific activity of the hen brain microsomal preparation was found to be 208.61 (±12.74 SD) nmole of phenol produced per minute per milligram protein at the tested conditions. The paraoxon-insensitive PV-hydrolyzing specific activity of the same preparation, the remaining activity after preincubation with paraoxon (40 μ*M*), was about 50% of the total activity [96.81 (±3.48 SD) nmole min^{-1} mg^{-1} protein].

Selective Inhibition of Paraoxon-Insensitive PV-hydrolyzing Activity

The *in vitro* effects of different concentrations of either mipafox or leptophosoxon on the remaining hen brain microsomal paraoxon-insensitive PV-hydrolyzing activity are

presented in Figure 1. Results indicated that the *in vitro* effects of these two neurotoxic compounds on that activity are parallel. About 67% to 70% of the remaining paraoxon-insensitive PV-hydrolyzing activity of the hen brain microsomal preparations was sensitive to either mipafox or leptophosoxon, while 33% to 30% of that activity was not sensitive to these neurotoxicants, even with relatively high concentrations. Increasing concentrations of mipafox from 1.0 to 40.0 μM and leptophosoxon from 0.02 to 0.50 μM, produced concentration dependent effects on that paraoxon-insensitive activity as shown in Figure 1. On the other hand, increasing the concentrations of either mipafox or leptophosoxon from 40 to 70 μM or from 0.5 to 0.7 μM, respectively, did not produce more inhibition of the remaining resistant activity. The difference between the paraoxon-insensitive and paraxon plus neurotoxicant-resistant activities was calculated to be the neurotoxicant-sensitive esterase activity (NTSE) or NTE.

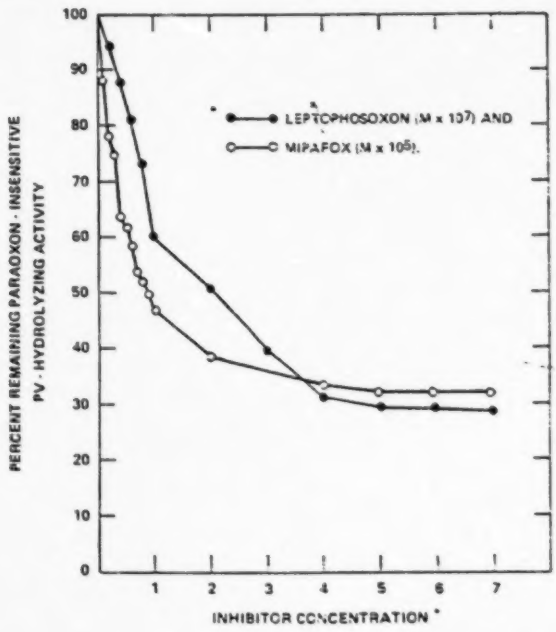
PERCENT REMAINING PARAOXON - INSENSITIVE PV - HYDROLYZING ACTIVITY
LEPTOPHOSOXON (M x 10⁷) AND
MIPAFOX (M x 10⁵).
INHIBITOR CONCENTRATION *

Figure 1. The remaining chicken brain microsomal PV-Hydrolyzing activity in presence of paraxon (40 μM) plus different concentrations of either mipafox or leptophosoxon.

Inhibition of NTE Activity by Mipafox and Leptophosoxon

The *in vitro* inhibitory effects of mipafox and leptophosoxon against the hen brain microsomal NTE activity are presented in Figure 2. The results show that leptophosoxon is more potent than mipafox as NTE inhibitor. In order to compare their inhibitory powers against NTE, the I_{50}, and I_{100} values, the concentrations required to inhibit 50% and 100% of the enzyme activity, respectively, were graphically estimated from Figure 2 and presented in Table I.

Leptophosoxon was 33.6 and 38.8 times as inhibitory as mipafox against hen brain NTE activity as calculated from their corresponding I_{50} and I_{100} values, respectively.

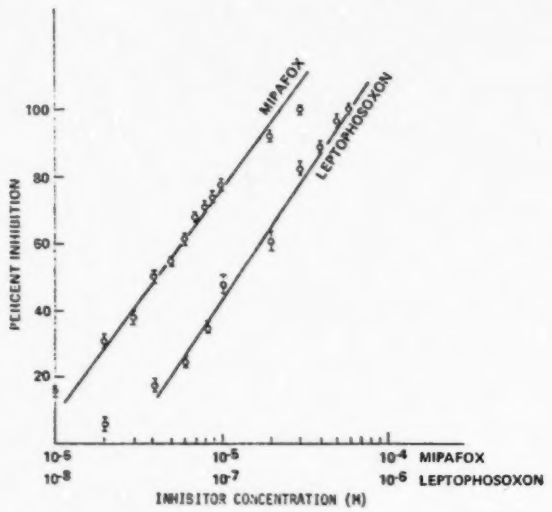

Figure 2. In vitro inhibition of chicken brain microsomal NTE activity by mipafox and leptophosoxon.

Table I. Inhibitory Effects of Mipafox and Leptophosoxon Against Chicken Brain Microsomal NTE Activity.

Neurotoxic Inhibitor	I_{50}^{*} (M)	I_{100}^{*} (M)	Remaining activity at I_{100}^{**} as a % of: Total activity	Paraoxon-insensitive activity
Mipafox	4.2×10^{-6}	2.3×10^{-5}	15~18	32~33
Leptophosoxon	1.25×10^{-7}	6.0×10^{-7}	13~15	29~31

*I_{50} and I_{100} are the concentrations required to produce 50 and 100% inhibition of the NTE activity respectively as obtained graphically from Figure 2.
**Accompanied with the presence of paraoxon (40 μM).

Discussion

Differentially, it is possible to divide the total hen brain microsomal PV-hydrolyzing activity into three categories. The first fraction of this activity is sensitive to the non-neurotoxic OP ester, paraoxon (40 μM), the second fraction is sensitive to neurotoxic agents and the third one is relatively resistant to high concentrations of the neurotoxic OP compounds. This remaining neurotoxicant-resistant activity, in the presence of both paraoxon (40 μM) and the tested neurotoxicant (I_{100}), as a percent of the total or the paraoxon-insensitive activities is presented in Table I. Results indicate that the same fraction of the hen brain microsomal PV-hydrolyzing activity is the target for either mipafox or leptophosoxon as shown in Figure 1 and Table I. This fraction, NTE, is about 34% or 35% of the total PV-hydrolyzing activity of the hen brain microsomal preparation when using mipafox or leptophosoxon as the selective inhibitor, respectively. These results are in agreement with that reported by Johnson (17,19), who found that the mipafox-sensitive PV-hydrolyzing activity was 37% of the

total and 65% of the paraoxon-insensitive activities. Moreover, the inhibitory effect of leptophosoxon against that fraction is much higher than that of mipafox. Leptophosoxon was also found to be relatively less hazardous, as a neurotoxic agent, than mipafox. The minimum effective doses of leptophosoxon and mipafox to produce neuropathy in hens were 100 and 25 mg/kg, respectively, as previously reported (23,24). Leptophosoxon is still commercially available as an experimental reagent or the same source from which we obtained it for this study (see materials). In addition, it is possible to convert leptophos [0-methyl-0-(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate; Phosvel®], quantitatively in solution into leptophosoxon by bromine oxidation (unpublished work). These findings suggest that leptophosoxon might be an alternative to mipafox as the selective NTE inhibitor under about the same conditions.

Acknowledgement

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